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SURVIVAL UNDER STRESS OF HALOTOLERANT LACTOBACILLI WITH PROBIOTIC PROPERTIES

SUPERVIVENCIA BAJO CONDICIONES DE ESTRÉS DE LACTOBACILOS HALOTOLERANTES CON CARACTERÍSTICAS PROBIÓTICAS

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Abstract

Three halotolerant lactobacilli with probiotic potential previously isolated from Chiapas cheese (*Lactobacillus plantarum*, *L. pentosus* and *L. acidipiscis*) and two commercial lactobacilli with probiotic activity (*L. casei* Shirota and *L. plantarum* 299v) were evaluated for their safety and survival capacity under stress. All the strains could grow in optimal conditions up to 6 % NaCl and showed sub-lethal growth up to 16 % NaCl; all the strains could grow well at pH values between 4.0 and 8.0; with a sub-lethal growth up to pH values of 2.0 and 9.0. *L. plantarum* 299v could grow up to 2.0 % of bile salts, and *L. acidipiscis* up to 1.5 % of bile dried salts. All the strains could be considered safe because all of them were γ-hemolytic and gelatinase negative. Moreover, all the strains showed similar antibiotic resistance pattern and resisted the normal dose used in the food industry of nisin and lysozyme. With these results, it is possible to conclude that the two commercial *Lactobacillus* strains are halotolerant and that all the strains can be used in a wide range of food products.

Keywords: probiotics, halotolerant lactobacilli, osmotic stress, pH stress, bile salt stress, antibiotic resistance, non-conventional preservatives.

Resumen

Tres cepas de lactobacilos halotolerantes con potencial probiótico previamente aislados del queso de Chiapas (*Lactobacillus plantarum*, *L. pentosus* y *L. acidipiscis*) así como dos lactobacilos comerciales con actividad probiótica (*L. casei* Shirota y *L. plantarum* 299v) fueron evaluados para determinar su seguridad y supervivencia bajo condiciones de estrés. Todas las cepas crecieron en óptimas condiciones al 6% NaCl y mostraron un crecimiento subletal hasta el 16% NaCl. Todas las cepas crecieron bien a valores de pH entre 4.0 y 8.0, con un crecimiento subletal hasta pH 2.0 y pH 9.0. *L. plantarum* 299v creció hasta con un 2.0 % de bilis deshidratada y *L. acidipiscis* hasta con un 1.5 %. Todas las cepas pueden considerarse seguras ya que resultaron γ-hemolíticas y gelatinasa negativas. Además, todas mostraron patrones similares de resistencia a antibióticos. Finalmente, todas las cepas resistieron las dosis normalmente utilizadas en la industria de alimentos de nisina y lisozima. Con estos resultados se puede concluir que las dos cepas comerciales de lactobacilos son halotolerantes y que las cinco cepas pueden usarse en un amplio rango de productos alimenticios.

Palabras clave: probióticos, lactobacilos halotolerantes, estrés osmótico, estrés a pH, estrés biliar, resistencia a antibióticos, conservantes no convencionales.

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1 Introduction

Lactobacillus is the largest genus within the group of lactic acid bacteria (LAB). They are Gram positive rods belonging to the Lactic Acid Bacteria (LAB) group. Their phenotypic traits, such as obligate/facultative, homo/hetero fermentative abilities play a crucial role in souring raw milk and in the production of fermented dairy products like cheese and fermented milks (including probiotics). They are also normal inhabitants in the human gut (de Angelis and Gobetti, 2004; Hamer and Hertel, 2006; Bernardeau *et al*., 2008). Lactobacilli confer a great economical value both in the pharmaceutical and the food industry due to this large amount of technological and physiological benefits (Deepika and Charalampopoulos, 2010; González-Olivares *et al*., 2011). Also, are commonly used both in traditional and industrial lactic acid fermentations because its contribution to conservation (due to the lactic acid, bacteriocins and hydrogen peroxide production) and to their capacity to contribute to their unique sensorial characteristics (Giraffa, 2012). In the last times, some strains of this genus have been recognized as probiotics since they give some health benefits to the host, when administered alive and in adequate amounts (Guarner y Saaftsa, 1998; Rodríguez-Huezo *et al*., 2011). Actually, besides of some strains of Lactobacilli and Bifidobacteria, some strains of non lactic acid bacteria and aerobic microorganisms (like *Bacillus claussii*) and even yeast (*Saccharomyces boulardii*) are considered as probiotic (Sánchez *et al*., 2013). Suitable probiotics should be selected according to one or more particular properties, being the preferred properties their competitive exclusion of pathogenic organisms from the surface to which they are applied, adherence to human tissue, sensitivity to antibiotics, antimicrobial activity, acid tolerance, and a high oxygen tolerance (Cinque *et al*., 2011). In fact, the high oxygen tolerance of probiotic strains is considered as a competitive advantage from probiotic microorganism because they are usually consumed in food products that contain dissolved oxygen like dairy products (Cesselim *et al*., 2011) and because it is an stress factor inside the gastrointestinal tract where there is a marked oxygen gradient from the stomach to the distal small intestine (He *et al*., 1999).

Lactobacilli are able to grow in a wide range of temperatures, salt concentration, oxygen tension and pH (Doyle and Meng 2006). This aspect is particularly important for the food industry since they are able to exert their beneficial effects over a wide range of technological conditions. In this aspect, osmotic and acid stress represents the major stress encountered by LAB during the fermentation of many food products such as cheeses. For example, salt, an osmotically active agent, varies in concentration from 0.7 to 6.0 % in cheeses and other food products (Guinee and Fox, 2004; Mayorga-Reyes *et al*., 2009) and pH in ranges from 2.0 in some kinds of vinegar to 9.0 in table olives. In addition to stress that bacteria support during their processing and storage, they find a hostile environment during their passage through the gastrointestinal tract (GIT) where pH that ranges from pH 2.0 to 9.0, bile salts reach concentrations of up to 2.0 % and osmotic pressure changes during the nutrient absorption in the gut (Beales, 2004; de Angelis, 2011). The survival of some *Lactobacillus* strains under the stressful conditions presents both in gastrointestinal tract and in food matrixes have been previously reported (Corcoran *et al*., 2008; Giraffa, 2012).

Low pH and high salinity limit the LAB strains growth when used as free cells (Ozer *et al*., 2008; Karimi *et al*., 2011). In addition, sodium chloride contributes to morphological changes in the cell free surface of *Lactobacillus* strains (Gong *et al*., 2012). Additionally, osmotic stress tolerance is an important factor in some food fermentations, acting as a key factor during the transit trough the GIT and where osmotic stress varies due to the peristaltic movement and the presence of water and food (Fordtran *et al*., 1965).

Non-starter LAB dominate cheese microbiota during ripening. They tolerate hostile environments and influence the curd biochemistry maturation, contributing to the final characteristics developed during the making process of the cheese. Several of these LAB have been selected on the basis of their health benefits and are employed in cheese making (Settanni and Moschetti, 2010). Probiotic LAB from the same species isolated from the human gut are actually used as non-starter LAB in some cheeses to improve their health benefits.

Another important aspect to consider is related with the ability to resist the presence of antibiotics, still a common contaminant in meat and dairy industries mostly in developing countries, and the preservatives used in food industry to avoid the appearance of spoilage and food borne microorganisms. Moreover, the use of combined treatments antibiotic - probiotic are under study to treat some diarrhea like those produced by *Clostridium difficile* (Mathur and Singh, 2005). Non-conventional

antimicrobials are used increasingly in meat and dairy products because of their high safety (Ramos-Villarroel *et al*., 2011) and the selection of LAB strains that can survive under the normal concentration usually found in these fermented foods are of interest. Finally, although most LAB usually employed as a starter or probiotics are from human of food origin, and were traditionally considered as safe, some diseases produced by *Lactobacillus* strains have been reported (Borriello *et al*., 2003). So it is convenient to guarantee their safety previously to their use (da Cunha *et al*., 2012; Husni *et al*., 1997) testing their virulence potential through some analysis as hemolytic and gelatinase actitivity (Singh *et al*., 2012; Giraffa, 2012).

Recently, three halotolerant strains from Chiapas cheese (*Lactobacillus plantarum*, *L. pentosus* and *L. acidipiscis*) has been isolated, characterized and identified (Morales *et al*., 2011); also, their probiotic potential has been proved including their *in vitro* survival to normal gastrointestinal conditions (through oral cavity to ileum) (Melgar-Lalanne *et al*., 2013). Chiapas cheese is a traditional raw milk cheese prepared in the tropical State of Chiapas (Mexico) with moisture of 48.2 %, pH of 4.03, salt concentration 5.34 % and a water activity (aw) of 0.972. On the other side, there are many lactobacilli considered as probiotics that are sold contained in food matrixes (as *L. casei* Shirota, Yakult ®) or as food supplements (as *L. plantarum* 299v, Protransitus®). These LAB are from human origin, and numerous studies have been conducted to ensure their effectiveness and safety (Sako, 2010) although no researches could be found to analyze their survival under stress conditions. Besides, no studies about their salt tolerance levels could be found to compare with the three halotolerant strains isolated from Chiapas cheese.

Halotolerance and origin of the strains may be two factors related with the survival of the strains under technological and physiological stressful conditions. In addition, the safety of the strains isolated from Chiapas cheese has not been previously reported.

For all the above, the purpose of the present research was to evaluate the bacterial growth under some technological and physiological stress conditions, of three halotolerant lactobacilli isolated from Chiapas cheese (*Lactobacillus plantarum*, *L. pentosus* and *L. acidipiscis*) and compared with two commercial probiotic strains from human origin (*L. casei* Shirota and *L. plantarum* 299v). Also, safety of halotolerant lactobacilli from Chiapas cheese and halotolerance of human origin *Lactobacillus* strains were evaluated.

2 Materials and methods

2.1 Bacterial strains and growth conditions

Three halotolerant strains previously isolated, identified and characterized from Chiapas cheese (Morales *et al*., 2011) were employed in the present research. Additionally, two commercial strains of *Lactobacillus* with probiotic activity from human origin were used as positive controls. Pure culture of the probiotic bacteria *Lactobacillus* casei Shirota was isolated from Yakult® (Yakult México, Mexico City, Mexico); meanwhile, *L. plantarum* 299v (Mendoza-Madrigal *et al*., 2013) was isolated similarly from Protransitus® (Laboratorios Salvat, Barcelona, Spain). All the LAB were incubated at 37°C for 24 h in lactobacilli MRS broth (Dibico, Mexico City, Mexico). Pathogenic strains of *Listeria monocytogenes* ATCC 19115 (ATCC, American Type Culture Collection, Manassas, VA, USA) and *Staphylococcus aureus* ATCC 25923 (ATCC, American Type Culture Collection, Manassas, VA, USA) were used as a control in safety assays when necessary. Pathogenic strains were grown in Müeller-Hinton broth (Difco, Detroit, USA) at 37°C under aerobic conditions. All the strains were preserved frozen at -20 °C (Whirpool WRT18AET, Mexico City, Mexico) with glycerol (Golden bell, Mexico CITY Mexico) as a cryoprotectant until used.

2.2 Halotolerance determination

Halotolerance was determined following the method described by Ciulla *et al*., 1994 with some modifications. Briefly, overnight cultures of *Lactobacillus* strains, both from human (*Lactobacillus casei* Shirota, *L. plantarum* 299v) and Chiapas cheese origin (*L. plantarum*, *L. pentosus* and *L. acidipiscis*), were analyzed for their salt tolerance capacity. Strains were inoculated at 5.0 % ($\sim 5 \times 10^7$ cfu/ml) in MRS broth (Difco, Detroit, MI, USA), with NaCl (Reasol, Mexico City, Mexico) concentrations ranged between 0.0 to 16.0 % (w/v) in 2.0 % intervals. Strains were incubated (Riossa, Mexico City, Mexico) at 32 °C for 24 h under aerobic and static conditions. Turbidity was measured with a spectrophotometer (Jenway 6405, Dunmow, Essex, UK) at 560 nm.

2.3 Acid and alkaline stress

Acid stress was determined following the method described by G-Alegria *et al*., 2004 with some modifications. *Lactobacillus* strains were prepared as above and then, inoculated at 10.0 % in MRS broth (~ 10^8 cfu/mL) with the pH adjusted between 2.0 to 9.0 using HCl 37 % (w/v) (Hycel, Jalisco, Mexico), or NaOH 45 % (w/v) (Hycel, Jalisco, Mexico). Strains were then incubated at 37 °C for 24 h under aerobic and static conditions (microaerobiosis) and final turbidity was measured by spectroscopy at 560 nm.

2.4 Bile and pancreatin tolerance

Tolerance to bile was assessed by studying the ability of strains to growth in the presence of different concentrations of bovine dried bile (Oxgall®, Sigma-Aldrich, St. Luis MO, USA) using modifications of the methods of Jamalli *et al*., 2011. Overnight cultures were centrifuged (3,000 ×g, 15 min, 4 °C), washed twice with Phosphate Buffered Saline (PBS) buffer (NaCl 8 g/L; KCl 0.2 g/L (Reasol, Mexico City, Mexico); $Na_2HPO_4 \cdot 2H_2O$ 1.44 g/L (Reasol, Mexico City, Mexico); KH_2PO_4 0.24 g/L (Reasol, Mexico City, Mexico); pH 7.2) and resuspended in the same buffer. After that, cells were inoculated at 10% in MRS broth (~10^8 cfu/ml) supplemented with bovine dried bile containing 0.3, 0.5, 1.0, 1.5 and 2.0 % (w/v); MRS without bile was considered as positive growth control. Tubes were incubated in a water bath at 37 °C for 24 h. absorbance was measured at at 24 h (the time that probiotics should survive adhered to the small intestine).

Tolerance to pancreatic fluid was tested basically with Rönka *et al*., 2003 methodology. *Lactobacillus* strains were prepared as above. After that, strains were centrifuged and washed twice with saline solution (9 g/L NaCl) and resuspended in the same solution. Bacteria (5.0 %) were inoculated in pancreatic medium (150 mM $NaHCO_3$ (Reasol, Mexico City, Mexico)+ 1.9 mg/mL L (Sigma, St. Louis, MO, USA), pH 8.0). The cultures were kept for 3 h in a shaking water bath (Thermo/Lab-Line/BRNAstead Max 4000, Artisan Scientific, Champaign, Illinois, USA) at 37 °C and 150 rpm. Survival, as colony forming unitis (cfu)/ml was examined by plating on MRS agar after 0, 1.5 and 3.0 h and saline solution was used as a control and results were expressed as Log cfu/ml.

2.5 Assays related to safety considerations

Hemolytic activity was studied following the methodology performed by Botes *et al*. (2008). Streaking fresh cultures on blood agar plate containing 10% (w/v) of human blood and incubating it at 37°C for 48 h. Blood agar plates were examined for signs of α-hemolysis (green-hued zones around colonies), β-hemolysis (clear zones around colonies), or γ-hemolysis (no zone around colonies) (Ambalam *et al*., 2013). *Listeria monocytogenes* ATCC 19115 (α-hemolytic) and *Salmonella enterica* ATCC 14028 (α and β hemolytic) were employed as positive controls.

Production of gelatinase was carried out using the method described by Botes *et al*. (2008). Overnight cultures of each *Lactobacillus* strain were streaked onto MRS agar supplemented with 3.05% (w/v) of gelatin (MCD Lab, Tlalnepantla, Mexico). Plates were incubated at 37°C for 24 h followed by incubation at 4°C for 5 h. After incubation, trichloroacetic acid (TCA) (Realitys Productos Químicos, Mexico City, Mexico) was added to the plate and observations were made at 5 min intervals (Medina and Barresi, 2007). Colonies with surrounding opaque zones were regarded as gelatinase positive. *S. enterica* ATCC 14028 was used as a positive control for gelatinase activity and *Listeria monocytogenes* ATCC 19115 was used as a negative control.

2.6 Antibiotic resistance test

The study of the resistance of *Lactobacillus* strains to twelve different antibiotics (tetracycline, ampicillin, erythromycin, cephalothin, cefuroxime, cefotaxime, penicillin, dicloxacillin, ceftazidime, sulfamethoxazole with trimethoprim, pefloxacin, and gentamicin) generally used against gram positive pathogens was performed by means of Multi-disks (Bio-Rad, Mexico City, Mexico) on MRS agar following the same methodology described by Jimenez-Serna and Hernández-Sánchez (2011). The minimum inhibitory concentration (MIC) of each antibiotic had been previously determined (Table 1) by the manufacturer and is the lowest concentration of the antimicrobial compound that inhibits the microorganism growth (Wikler, 2006).

2.7 Non conventional antimicrobials

Nisin is a bacteriocin frequently used as an antimicrobial in cheeses and other fermented foods for its safety and wide antimicrobial spectra. In Mexico,

the regulation (NOM-121-SSA1-1994) allows its use at concentrations up to 0.00125 % of nisin in processed cheese.

Nisin from *Lactococcus lactis* (subsp. *lactis*) and 10^6 IU / g (Sigma-Aldrich, St. Louis, MO, USA) was solubilized in 0.02 M HCl at a concentration of 10 mg / mL. The solution was filtered sterilized prior to use and added to vials (1 mL) to have concentrations of 50, 25, 12.5, 6.7, 3.35 and 1.85 μg nisin / mL in MRS broth. Subsequently, 1 μL of 10^8-10^9 ufc/mL of *Lactobacillus* strains previously incubated overnight were incubated at 37°C for 24 h. Turbidity was used as growth indicator to determine the minimal inhibitory concentration (MIC) of nisin (González-Sánchez *et al*., 2010). MIC is considered as the first transparent tube (showing no growth).

Lysozyme is a lytic enzyme used as GRAS food preservative in countries like USA and Mexico (Davidson y Critzer, 2012). To calculate the lysozyme MIC in the five strains of lactobacilli tested, the method suggested by Tribst *et al*. (2007) was followed with a few modifications. For that, inocula of 5.0 % during overnight cultures were added to tubes with MRS broth plus egg white lysozyme (0.0, 15.62, 31.25, 62.5, 125 y 250 μg lysozyme/mL (Sigma, St. Louis, Mo, USA). Tubes were incubated for 24 h at 30°C and microbial growth was measured by turbidity. As above, MIC of lysozyme was the minimum concentration able to inhibit the microbial growth at 24 h.

2.8 Statistical analysis

Results were expressed as the average of three independent experiments. Each experiment was subjected to one way analysis of variance (ANOVA) followed by a Student Newman Keuls test. Probability test ($P \leq 0.05$) was taken as criteria for significant difference, as indicated in each case. All statistical analyses were performed using MS-Excel® software and Sigma Plot 11.0 (SigmaPlot, SPSS Inc., IL; USA).

3 Results and discussion

3.1 Halotolerance

The determination of the halotolerance is of interest to know the type of food products to which they can be successfully added, and also to give a comprehensive idea of their probability to survive under the osmotic stress present in the GIT. Most of food salty products have salt concentrations lower than 6 %. However, some fermented products as soy and meat sausages may contain up to 18% NaCl (Rasmusen *et al*., 2010).

It is important to remark that halotolerance of *Lactobacilli plantarum*, *L. pentosus* and *L. acidipiscis* isolated from Chiapas cheese had been previously determined in glucose-yeast, extract peptone, meat-extract (GYPC) broth at 32°C during 24 - 48 h. However in order to better compare the results with the two human origin strains (*Lactobacillus casei* Shirota and *L. plantarum* 299v) it was decided to repeat this study using MRS broth (a more specific broth for lactobacilli) at different NaCl concentrations.

The five *Lactobacillus* strains tested showed different salt tolerance levels when incubated at 32°C for 24 h under aerobic conditions (Fig. 1). It could be observed that between 0 to 6 % NaCl concentrations all the strains showed a slightly reduction in their growth, between 6 to 10% NaCl all the strains drastically reduced their growth and between 10 to 16% NaCl all the strains could show a sub-lethal growth, bacteria could survive but not to grow maintaining their initial turbidity (*data not shown*).

The most sensitive strain to salt was *L. pentosus* from Chiapas cheese at 6.0 % and 16.0 % NaCl meanwhile the most resistant at 6.0 % was *L. plantarum* from Chiapas cheese, and at 16.0 % *L. casei* Shirota from human origin. However, no significant differences were found ($P > 0.05$) among behaviors to sodium chloride regardless their origin. Notably, the results obtained with the three halotolerant strains from Chiapas cheese were consistent with other previously reported (Morales *et al*., 2011). No reports about *L. casei* Shirota and *L. plantarum* 299v halotolerance were found. The five strains of *Lactobacillus* can be considered slightly salt tolerant (Larsen, 1986) because they could grow with few limitations until 6.0 % NaCl concentration. To our knowledge this is the first time that halotolerance from probiotic bacteria *L. casei* Shirota and *L. plantarum* 299v has been documented. So that, the five strains tested might grow in salty cheeses as Chiapas cheese.

3.2 Acid and alkaline tolerance

Lactobacilli survival at different pH values is important both at technological and physiological levels. In the stomach, pH ranges from 1.5 to 2.0 under fasting conditions and from 8.0 to 9.0 in the small intestine under satiety conditions. A similar pH span can be found in fermented foods (from pH 2.0 in vinegar to pH 8.0-9.0 in olives and some African and

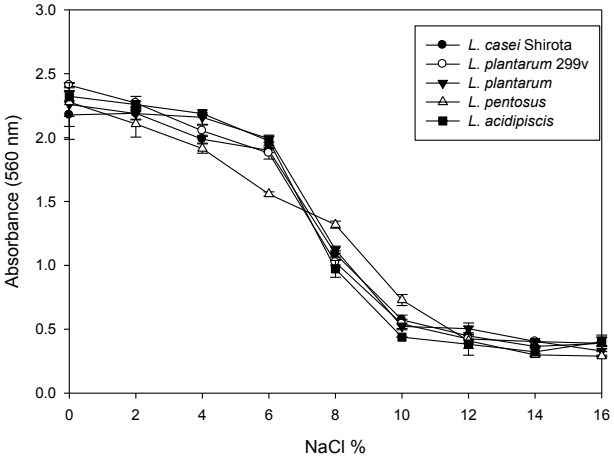

Fig. 1. Absorbance at 560 nm after 24 h of incubation at 32°C in the presence of different NaCl concentrations of *Lactobacillus* strains isolated from Chiapas cheese (*Lactobacillus plantarum*, *L. pentosus* y *L. acidipiscis*) and from human origin (*L. casei* Shirota y *L. plantarum* 299v).

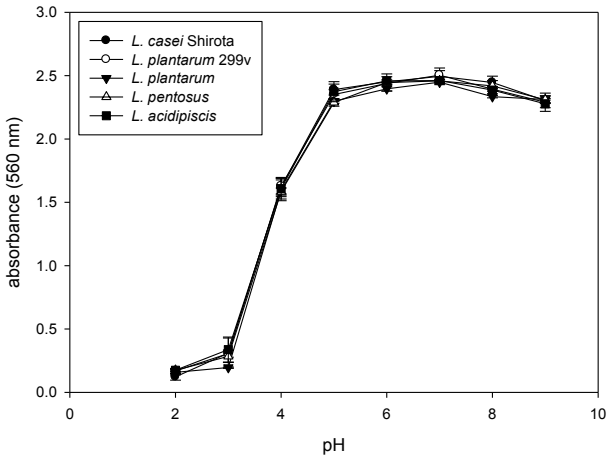

Fig. 2. Absorbance at 560 nm after 24 h of incubation at 37 °C under acid and alkaline conditions (pH ranged from 2.0 to 9.0) of *Lactobacillus* strains isolated from Chiapas cheese (*Lactobacillus plantarum*, *L. pentosus* y *L. acidipiscis*) and from human origin (*L. casei* Shirota y *L. plantarum* 299v).

Asiatic traditional foods) (Wang *et al*., 1996 Ouoba *et al*., 2010).

Acid and alkaline tolerance of the three strains of lactobacilli isolated from Chiapas cheese (*Lactobacillus plantarum*, *L. pentosus* and *L. acidipiscis*) and the two strains obtained from the probiotic lactobacilli from human origin (*Lactobacillus casei* Shirota and *L. plantarum* 299v) were analyzed (results are shown in Fig. 2).

The five halotolerant lactobacilli, no matter their origin, showed a similar behavior under the different pH tested. The most stressful conditions for the five *Lactobacillus* strains were pH 2.0 where they could not grow (showing a sub-lethal growth) due to the extremely acidity. At pH 3.0, all the strains could survive and even started to grow. At H 4.0 all the strains could grow and between pH 5.0 to 9.0 all the strains showed a good growth. The good growth at pH 9.0 indicated that the strains were able to survive under the small intestine conditions. The capacity to survive under the most stressful conditions (pH 2.0 and pH 9.0) indicated that the five strains are able to survive under the pH conditions usually found inside the gastrointestinal tract as a previously reported (Melgar-Lalanne *et al*., 2013). Also, the high growth between pH 4.0 to 9.0 has possible for these strains to be present in a large number of fermented foods.

Similar results under acidic conditions were found in other researches with *L. plantarum* strains (Bao *et al*., 2012) but, no references could be found about the growth of lactobacilli strains between pH 2.0 to 9.0.

3.3 Tolerance to bile and pancreatin

Bile salt resistance is essential for colonization in the small intestine of probiotic bacteria to secrete beneficial metabolites (Darilmaz y Beyatli, 2012).

Normal bile salt concentrations inside the inside the small intestine is between 0.3 to 0.5%. However, this concentration are directly related with the fat intake and concentrations up to 3.0% could be found (Kailassapathy and Chin 2011; Whitehead *et al*., 2008). So, to better understand the bile tolerance process, a longer time (24 h) was chosen because it is expected than probotics survive adhered to the intestine more time than the physiological transit time, and the capacity of adaptation to the presence of bile salts is related with some physiological benefits like the serum cholesterol reduction (Haman *et al*., 2011). However, the ileum and dudodeum simulation (under normal conditions of bile salts and pancreatin) was previously reported for these strains showing a great bile salt tolerance under normal physiological conditions (5 mg/ml bile salt plus 1.9 mg/ml pancreatin during 3 h) (Melgar *et al*., 2013).

The five *Lactobacillus* strains tested could grow at the maximum bile salt concentration tested (3.0%) at 24 h (Fig. 3).

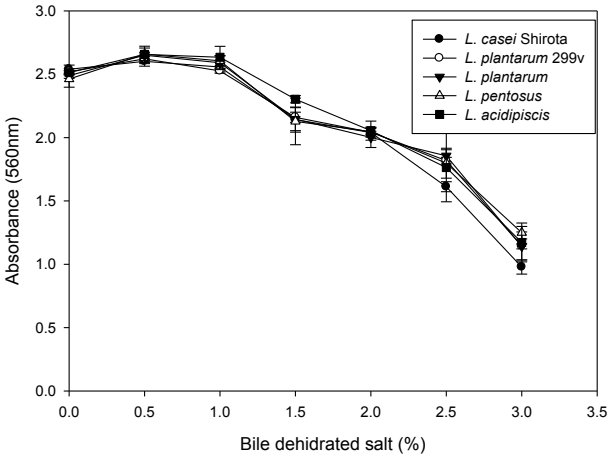

Fig. 3. Absorbance at 560 nm after 24 h of incubation at 37 °C at bile salt concentration between 0.0 to 3.0 % of *Lactobacillus* strains isolated from Chiapas cheese (*Lactobacillus plantarum*, *L. pentosus* y *L. acidipiscis*) and from human origin (*L. casei* Shirota y *L. plantarum* 299v).

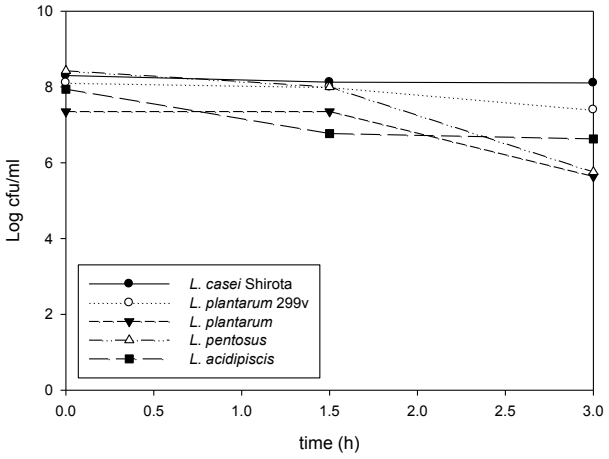

Fig. 4. Log cfu/mL in the presence of to pancreatin (1.9 mg/mL) of *L. casei* Shirota, *L. plantarum* 299v, *L. plantarum*, *L. pentosus* and *L. acidipiscis* incubated at 37°C and 150 rpm at 0, 1.5 and 3 h.

All the strains showed a similar behavior and could grow until 1.0% of bile salt and their growth was reduced between 1.0 to 3.0% of bile salt. No correlation was found between sodium chloride tolerance and bile salts tolerance ($p \geq 0.05$), so these two factors are independent for the strains tested. Results obtained with the two *Lactobacillus plantarum* strains agreed with what other researchers reported in similar conditions for other *L. plantarum* strains. Zago *et al*. (2011) analyzed the absorbance (600 nm) of 27 *L. plantarum* strains up to bile salt concentrations of 1.0% at 371C after 24 h and concluded that the bile salt resistance was variable and depended of the strain tested. A similar conclusion was reported by Jamali *et al*. (2011). Moreover, the high bile salt tolerance of halotolerant lactobacilli analyzed is consistent with previously reported for other potential probiotic strains (Vinderola and Reinheimer, 2003) who found a higher survival of potential probiotic strains against lactic acid starter. So that, probiotic and potential probiotic halotolerant lactobacilli could survive under the maximum stress conditions present in the small intestine when excessive fat is consumed in the diet.

Pancreatin is a mixture of several digestive enzymes produced by the exocrine cells of the pancreas through the small intestine and is mainly composed of amylase, lipase and trypsin. So, it is a stressful condition for bacteria that needs to adhere to the intestinal epithelium. The two lactobacilli from human origin (*L. casei* Shirota y *L. plantarum* 299v) were the most resistant strains to pancreatin at 3 h. Nonetheless, all the strains could survive under the stressful conditions caused by pancreatin (Fig. 4). Human origin strains showed higher resistance to pancreatin possibly due to the high concentration of digestive enzymes found in the human gut.

At the same time, it is also important to observe that in the intermediate count at 1.5 h, the strain with the best survival was *L. plantarum* from Chiapas cheese. The most sensitive strain was *L. acidipiscis*. However, this strain was able to survive thought the 3 h of the experiment. Results obtained with *L. casei* Shirota and *L. plantarum* strains are consistent with those reported in the literature for other strains of these LAB (Bertazzoni *et al*., 2004, Yu *et al*., 2013), indicating the consistency of the results showed previously.

3.4 Safety considerations

There is a general consensus that the safety of new strains intended for biotechnological (human and animal) use, has to be demonstrated (Ji *et al*., 2013). In recent years, strains of the genus *Lactobacillus* have been isolated in extremely rare occasions from infective lesions and clinical specimens. Even when uniformity in test procedures and safe criteria has not been well established worldwide, the safety

characterization of new strains generally focuses on features such as hemolytic and gelatinase activity (Sanders *et al*., 2010) which were also used for this study. Results indicate that the five halotolerant lactobacilli tested are safe because no hemolytic or gelatinase activity was found. In the case of hemolytic activity, the reliability of the test was confirmed by *Listeria monocytogenes* ATCC 19115 (α-hemolityc) and *S. enterica* ATCC 14028 (α and β hemolytic) as positive controls. Meanwhile, in the case of the gelatinase activity assay, *S. enterica* ATCC 14028 was used as a gelatinase positive strain.

3.5 Antibiotic resistance

Antibiotic resistance is a public health concern worldwide. In some lactobacilli, the resistance to chloramphenicol, erythromycin and tetracycline is associated with genes existent in transmissible vectors and a horizontal gene transfer between the probiotic and pathogen strains inside the gut is possible (Rabia and Shah, 2011). On the other hand, when antibiotic resistance is not associated to transfer vectors, it could be a desirable characteristic in the development of combined therapies (antibiotic / probiotic), which are successfully researched for diarrhea control (Mathur and Singh, 2005).

The five strains tested showed different antibiotic resistance patterns (resistance to 3 antibiotics in *L. casei* Shirota and to 7 in *L. plantarum* 299v and *L. pentosus*). *L. plantarum* 299v from human origin was resistant to 7 antibiotics, but *L. plantarum* might be associated to strain level (Nawaz *et al*., 2011). The antibiotic resistance of the five strains of lactobacilli tested is shown in Table 1.

All the five strains tested were resistant to gentamicin (an aminoglycoside antibiotic) and sensitive to ampicillin (a β-lactamic). The results obtained with commercial probiotics were similar to those of other researchers (Jiménez-Serna and Hernández-Sánchez, 2011; Ji *et al*., 2013). Moreover, results were also similar to those obtained with others *L. plantarum*, *L. pentosus* and *L. acidipiscis* strains (Argyry *et al*., 2013; Luo *et al*., 2012).

3.6 Non-conventional antimicrobials

As previously explained, nisin and lysozyme are two antimicrobials used currently in the food industry because of their documented safety and pathogenic antimicrobial spectra. (Ramos-Villarroel *et al*., 2010).

Nisin is a polycyclic antibacterial peptide with 34 amino acid residues (a bacteriocin) produced by some strains of *Lactococcus lactis* subsp. *lactis* and commonly used in cheeses. In Mexico, the maximum dose allowed is 12.5 mg of nisin / kg (NOM-121-SSA1-1994) for processed cheeses. In the five *Lactobacillus* strain tested, the MIC of nisin was between 25 and 50 μg nisin / mL (Table 2).

Table 1. Minimum Inhibitory Concentration (MIC) of ampicillin, cephalotin, cefoxatime, ceftazidime, cefuroxime, dicloxacilin, erytromycin, gentamicin, pefloxacin, penicillin, tetracycline and trimethoprim-sulfamethoxazole in *Lactobacillus casei* Shirota, *L. plantarum* 299v, *L. acidipiscis*, *L. plantarum* y *L. pentosus*.

Antibiotic	MIC (a)	*L. casei* Shirota	*L. plantarum* 299v	*L. plantarum*	*L. pentosus*	*L. acidipiscis*
Ampicillin	10 μg	S	S	S	S	S
Cephalotin	30 μg	S	R	I	R	I
Cefoxatime	30 μg	S	I	R	I	S
Ceftazidime	30 μg	S	R	R	R	R
Cefuroxime	30 μg	S	I	I	R	I
Dicloxacilin	1 μg	S	R	R	S	S
Erytromycin	15 μg	I	R	I	I	R
Gentamicin	10 μg	R	R	R	R	R
Pefloxacin	5 μg R	R	I	R	R	
Penicillin	10 U S	S	S	R	R	
Tetracycline	30 μg	S	R	S	R	R
Trimethoprim-sulfamethoxazole	25 μg	R	S	S	I	S

(a) MIC of each antibiotic determined by Wikler (2006). S: sensitive, R: resistant y I: intermediary sensibility.

Table 2. Minimum inhibitory concentration (MIC) of nisin and lysozyme in *L. casei* Shirota, *L. plantarum* 299v, *L. plantarum*, *L. pentosus* and *L. acidipiscis* strains.

Strain	MIC nisin (μg / mL)	MIC lysozyme (μg / mL)
L. plantarum 299v	50	250
L. casei Shirota	50	250
L. plantarum	50	250
L. pentosus	50	250
L. acidipiscis	25	250

Per se, the five strains could be used into the maximum permissible limit in Mexico without affecting their survival. Moreover, in Chiapas cheese, as in other traditional and homemade cheeses, the only possible source of nisin is the endogenous production of this bacteriocin by some strains of *Lactococcus lactis* subsp. lactis. In these conditions, this production could not reach the maximum allowable limit (Sobrino-López and Martín-Belloso, 2008). These results are consistent with those reported for other *L. casei* strains (Breuer y Radler, 1996) and *L. plantarum* and *L. pentosus* strains (Rojo- Bezares *et al*., 2006) showing a complete compatibility of the strains tested with starter cultures.

Lysozyme is used as a cheese preservative in the European Union as well as in the USA without a legal limit because it is considered GRAS (Davidson and Critzer, 2012). All the *Lactobacillus* strains tested survived the maximum MIC analyzed (250 μg of lysozyme / mL. So, these results showed that the five strains tested could be used without restriction in food products in the presence of lysozyme as in the case of cheeses.

Results are consistent with those reported for *L. casei* Shirota (Rodríguez *et al*., 2012) and for various *L. plantarum* strains (Zago *et al*., 2011). Intrinsically, all the strains tested in the present research have a similar behavior as other LAB.

Conclusions

The five strains of commercial and potential probiotic lactobacilli tested could survive under different physiological stressful conditions (pH, bile salt and pancreatin). Also, because their ability to grow or survive under different pH (from 2.0 to 9.0) as well as their resistance to the presence of non-conventional antimicrobials they could be used in many food products in a safe way. Finally, the two commercial probiotics (*Lactobacillus casei* Shirota and *L. plantarum* 299v) showed halotolerant characteristics similar to that of the halotolerant lactobacilli isolated from Chiapas cheese (*L. plantarum*, *L. pentosus* and *L. acidipiscis*) and could be considered as halotolerant.

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